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PRINCIPAL INVESTIGATOR: J. W. Chiao, PhD

CONTRACTING ORGANIZATION: New York Medical College  
Valhalla, NY 10595

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14. ABSTRACT: There are epidemiological conclusions for potential prevention of prostate cancer with the intake of cruciferous vegetables. The responsible dietary factors were not identified. Now, we have demonstrated that isothiocyanates, such as phenethyl isothiocyanate (PEITC), from these vegetables inhibits growth of prostate cancer cells by targeting cell cycle regulators, like up-regulating p21. Analyses of the mechanism indicated that it involves a regulation at the epigenetic level. PEITC was found to be a dual inhibitor of histone deacetylases (HDACs) and aberrant CpG island methylation. Due to HDAC inhibition, PEITC modifies histones for transcription competent chromatin to activate p21. We demonstrated, for the first time, that PEITC reverses hypermethylation and reactivates GSTP1 that is silenced in prostate tumor. The silencing of GSTP1 occurs in the vast majority of clinical tumors and is a risk factor. We also determined that PEITC represses androgen receptor (AR) via down-regulation of Sp1 and increase protein degradation. The bioassay showed that PEITC regulates the activity of testosterone, via down-regulation of AR. Thus we have demonstrated several molecular targets relevant for inhibiting prostate carcinogenesis by PEITC. They include repressing AR, inhibiting HDACs, inhibiting aberrant CpG island methylation, and activating cell cycle regulators. The chemopreventive mechanisms of isothiocyanates revealed.					
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## Introduction:

Our research objectives are to investigate phenethyl isothiocyanate (PEITC), found rich in watercress (cruciferous vegetable), as a potential dietary factor for prevention of prostate cancer. The hypothesis is that PEITC induces growth arrest in prostate tumor cells thereby inhibiting post-initiation progression of prostate carcinogenesis.

Since we made the hypothesis that isothiocyanates and therefore cruciferous vegetables are potential dietary factors for prostate cancer chemoprevention (8), there have been conclusions from epidemiological reports that consumption of cruciferous vegetables is inversely related to prostate cancer incidence. We have since determined that isothiocyanates may represent a chemical group with potential effects to inhibit the initiation and progression of prostate cancer. They target the cell cycle regulators to induce cell cycle arrest, in culture and in xenografts. Since the reports of our original findings, there has been numerous publications from other investigators, regarding the effects of isothiocyanates regulating the growth of prostate cancer cells.

Recently we have reported several new mechanisms, for the first time, as the basis of growth arrest induced by PEITC. These mechanisms target the critical events of prostate cancer pathogenesis, and are reported in the following.

## Body

Tasks 2 and 3: Perform experiments with human prostate cancer cell lines *in vitro* to examine the effects of PEITC-investigation of growth regulation, cell cycle progression, p21 induction and the mechanisms.

### **1) Cell cycle effects--PEITC induces inhibitors of cyclin dependent kinases and reduces Rb for cell cycle arrest:**

We demonstrated that exposure of human prostate cell lines to PEITC, including LNCaP and DU-145, induced G1 arrest and apoptosis (1, 6, 8). The expression of the inhibitors of cyclin dependent kinases (cdks), such as p21, during G1- to S-phase transition was induced, which is related to PEITC concentrations and independent of p53 (Figure 1) (1, 5). The enhanced levels of cdk inhibitors could increase their binding with the effector cyclin/cdk complexes. As the result, the Rb proteins, executed by the cdk activity to activate G1- to S-phase transition, diminished (Figure 1).

### **2) Targeting cell cycle arrest by an epigenetic regulation--PEITC as an inhibitor of histone deacetylases (HDACs) that activates p21:**

We discovered that PEITC is an inhibitor of HDACs that are enzymes responsible for removing acetylation on histones. Exposure of HDAC enzymes to PEITC in a cell-free assay revealed a concentration-related inhibition of the enzyme activity (Figure 2B) (4). Exposure of prostate cancer cells LNCaP to PEITC inhibited the HDAC activity in LNCaP cells. The magnitude of inhibition relates to the concentrations of PEITC (Figure 2C). Inhibition of HDAC activity

results in an enhanced histone acetylation. The status of histone acetylation was evaluated, and PEITC-exposed prostate cancer cells exhibited significant enhancement of global acetylation of histones (Figure 2). Figure 2A also shows that PEITC down-regulated the level of HDACs, supporting the finding of a lowered HDAC activity in the PEITC-exposed prostate cancer cells. PEITC additionally altered selective histone methylation (Figure 2C). The altered patterns of histone acetylation and methylation, mediated by PEITC, are consistent to that of chromatin unfolding, for easy access for the transcription factors to the associated genes.

These findings provided the rationale to examine the relationship between p21 activation by PEITC and acetylated histones. The chromatin immunoprecipitation (ChIP) assay was performed, employing an anti-acetylated histone H3 antibody to precipitate the chromatin fragments from untreated LNCaP cells, or from PEITC-exposed LNCaP cells. As shown in Figure 3, the chromatin from cells exposed to PEITC clearly contained the p21 gene fragments; as compared to the control untreated LNCaP cells where p21 was nearly undetectable (1). The results clearly indicated an association of the p21 gene with the hyperacetylated histones induced by PEITC, and the resulting transcriptional active chromatin allowed the activation of p21.

### **3) Targeting androgen receptor (AR) by PEITC for growth attenuation in prostate cancer cells.**

The androgen receptor (AR) is a pivotal factor for androgen-mediated growth and maintenance of the prostate. Abnormality of the AR, including over-expression, is implicated in the initiation and progression of prostate cancer. We demonstrated that PEITC induced a significant growth inhibition with equal activity in androgen dependent (AD), and –independent (AI) prostate cancer LNCaP lines (9). In the presence of PEITC, the AR present in both cell lines was repressed as demonstrated with real-time PCR and Western blot (Figure 4) (9). Since Sp1 drives AR transcription, we examined that the repression of AR by PEITC could be mediated by an inhibition of Sp1. We tested the effects of PEITC on the activities of Sp1-luc, containing three repeats of Sp1 consensus sites, or its mutant mSp1-luc. After exposure of LNCaP cells to mithramycin, a specific Sp1 inhibitor caused a strong inhibition of the Sp1-luc activity whereas trichostatin A (TSA) mediated a strong induction (Figure 5A). PEITC mediated a significant inhibition of Sp1-luc activity in both AD and AI cells (Figure 5B). As is also revealed, PEITC caused reduction of Sp1 protein level, which associated with the decrease of Sp1 activity (Figure 5C) (9).

To further examine whether the suppression of the AR and Sp1 expression by PEITC is a result of proteasome-dependent protein degradation, the AD cells were exposed to PEITC, protein synthesis inhibitor cycloheximide (CHX), or proteasome inhibitor MG-132 alone, or to the combination of PEITC plus CHX or MG-132. As shown in figure 6, the PEITC inhibitory effects for Sp1 was abrogated by the presence of PEITC plus either CHX or MG-132. The results indicated that down-regulation of Sp1 may require protein synthesis for a proteasome activity, thereby enhancing Sp1 protein degradation.

To summarize, at transcriptional level the AR level was reduced via inhibition of the transcription factor Sp1, and at post-translational level by accelerating protein degradation. We conclude that PEITC targets AR over expression to modulate the growth of androgen-dependent

and -independent prostate cancer cells. It is an important mechanism relevant in preventing the initiating and inhibiting the progression of prostate cancer.

#### **4) PEITC targets inhibiting aberrant DNA methylation in prostate cancer cells as a mechanism of chemoprevention and tumor elimination.**

Prostate cancer is characterized with the loss of expression of a major detoxifying enzyme, the  $\pi$ -class glutathione S-transferase (GSTP1), due to aberrant CpG island methylation. Greater than 95% of clinical tumors lack GSTP1, and is considered as a risk factor for the disease. The loss of GSTP1 is a diagnostic and pathologic marker, and probably an underling cause of oxidative damage and inflammation at tumor initiation.

We reported that exposure of LNCaP AD (androgen dependent) or AI (androgen independent) cancer cells to PEITC resulted in reactivation of GSPT1. Analyzed with methylation specific PCR, the CpG island of the GSTP1 gene was demethylated by PEITC and the gene unmethylated at CpG island reappeared (Figure 6). Upon further quantification by pyrosequencing, the methylation level of the GSTP1 after PEITC exposure was reduced to that found in normal prostatic cells (4, 7). PEITC inhibits the aberrant DNA methylation of GSTP1 gene concurrently with inhibition of HDACs. The dual action of PEITC on the DNA and histones may de-repress the methyl-binding domain on gene transcription. As a result, the expression of GSTP1 was recovered in the prostate cancer cells, and the detoxifying function to remove toxins was restored in the prostate cancer cells (4). Reactivation of genes by PEITC allows the tumor cells revert to a more normal state, and the recovered cellular functions could overcome the tumorigenesis.

Unlike the known inhibitors for DNA methylation or HDACs that inhibit methylation or HDACs but not both, PEITC inhibits both DNA methylation and HDACs concurrently. This may mediate synergistic effects, representing a novel function for epigenetic regulation. There are epigenetic therapies using HDAC inhibitors, DNA methylation inhibitors, or their combination in clinics. The effects of PEITC to target HDACs and the aberrant methylation at CpG islands are relevant mechanisms for preventing and treating prostate cancer.

Judged together with our data, PEITC regulates cell cycle progression with an up-regulation of the kinase inhibitors. This may also relate to the repressing of AR in the prostate cancer cells, therefore the uncontrolled growth is inhibited. These data have indicated that the epigenetic regulation including the inhibition of HDACs, may represent the primary mechanism of growth regulation of prostate cancer cells.

#### **5) Isothiocyanates other than PEITC can induce growth arrest and apoptosis in prostate cancer cells--isothiocyanates and cruciferous vegetables are chemopreventive and chemotherapeutic agents.**

To evaluate whether the growth regulatory effects of PEITC is common with other isothiocyanates that are present in the variety of cruciferous vegetables, we have examined the effects of phenylhexyl isothiocyanate (PHI). PHI was also determined to be an inhibitor of HDAC activity (2). Exposure of prostate cancer cells LNCaP to PHI induced G1 arrest and

apoptosis. Studies of mechanism indicated that PHI inhibits HDACs and enhanced acetylation of histones. As a result, the chromatin unfolding caused activation of genes like p21, which is associated with hyperacetylated histones (2, 10).

We also examined whether isothiocyanates other than PEITC are chemopreventive and therapeutic agents for other types of cancer. We have demonstrated that PHI induces G1 arrest and apoptosis in leukemia cells in culture and in xenografts (10, 11). Studies showed the same mechanism as that seen with the prostate cancer cells that PHI inhibits HDACs and induced hyperacetylation of histones, leading to chromatin unfolding and activated p21, for cell cycle G1 arrest.

These analyses are important to reveal that isothiocyanates from cruciferous vegetables are the responsible dietary factors for preventing and inhibiting prostate cancer, and most likely effective for other types of cancer as well.

Tasks 3-6. Analyses of the effects of PEITC on normal prostatic cells, the effects on cell proliferation, and the *in vivo* effects of PEITC in the rat prostate tumors.

#### **1) PEITC regulates testosterone-mediated growth of the normal prostates--a basis of prostate cancer prevention.**

The influence of oral feeding of PEITC on the growth of the normal prostates, stimulated by testosterone was analyzed in the rats. To reduce the effect of endogenous androgen on the prostates, rats were first provided with cyproterone acetate for 5 days/week for 3 weeks. Rats were given SC injections of testosterone propionate every 3 days for a total of five injections. These rats were then randomly divided into two groups, with the first group receiving by gavage PEITC prepared in corn oil, at 12  $\mu\text{mol/rat}$  (80% MTD), every other day for 3 weeks, starting from the first day while receiving cyproterone acetate until 2 weeks after the last dose of testosterone. The second group of rats received corn oil as a vehicle control. A separate third group as a normal control received corn oil without cyproterone acetate, testosterone, and PEITC.

After the PEITC treatment for 3 weeks, the averages of the body weight of these three groups of rats were similar, and the PEITC feedings to the rats showed no overt toxicity. The weight of the prostates, after priming of testosterone, showed a regular increase from the untreated control rats (Figure 8). After feeding PEITC, the weight of the prostates was significantly lowered, in comparison to the testosterone control (30% decrease,  $P=0.05$ ), and was almost the same weight as the normal rats (3). The prostate weight of the untreated control rats and the PEITC-treated group was similar and the difference was not significant (Fig 8).

While testosterone increased the prostate mass and hyperplastic seminiferous tubules, as compared to the untreated rats, PEITC feeding decreased the prostate mass and hyperplasia to the levels of untreated rats ( $P<0.05$ ). PEITC negated the testosterone-mediated enhancement of the AR, via down-regulating transcription factor Sp1 expression (Figure 9) and the Sp1 binding complex formation (3). Cell cycle progression was attenuated with decreases of cyclins, cdks, Rb phosphorylation, and up-regulation of cdk inhibitor p27 (Figure 10).

Our conclusion is that oral feeding PEITC to the rats does not cause overt toxicity. PEITC from cruciferous vegetables may represent a regulator *in vivo*, for the androgen-dependent growth of the prostates. These data support our conclusion that repressing AR is a target of PEITC, which is relevant in prostate cancer chemoprevention.

## **2) The *in vivo* effects of PEITC.**

With the task of a bioassay with WU rats, the rats were primed with antiandrogen flutamide, then were provided with testosterone propionate and followed by an injection of carcinogen N-methyl-N-nitrosourea (MNU) for the formation of prostate tumors. The experimental group of rats was provided with PEITC, to evaluate the response on carcinogen-induced tumor.

After MNU injection, the numbers of rats developed prostate tumors were much lower than that reported in the literature. Since there were tumors of different differentiation status, the number of rats in each pathology grouping was also small. After consulting with experts on this WU rat prostate cancer model, the low number of prostate tumor is likely due to either the MNU we purchased were not active, or an insufficient amount of MNU was injected into the rats.

## **3) Induction of growth modulation and apoptosis in rat prostate tumors**

Cellular proliferation was compared between the tumor-bearing prostates from untreated rats and the prostates from rats received PEITC feeding as a treatment. The expression of PCNA, a proliferating cell indicator for S-phase cell cycle, was significantly lower in the PEITC-treated prostatic cells than the untreated control prostates (Figure 11). This has indicated a reduced proliferation and growth of the prostate tumors with the PEITC treatment. Figure 11 also shows a cleavage of PARP, which is a hallmark of apoptosis that became undetected in the PEITC-treated prostates. The reduced expression of PARP is in line with the detection of increased apoptosis in the PEITC-treated prostates. To examine the mechanisms related to growth regulation, the status of histone acetylation was evaluated. The global acetylation of histone H3 and selective acetylation of H3k14 was clearly enhanced in the prostates of PEITC-treated rats, as compared to the untreated controls (Figure 11). This has indicated a chromatin remodeling in the rat prostates by the PEITC treatment. These results indicated that PEITC may mediate histone acetylation, via HDAC inhibition, leading to activation of the hyperacetylation-associated genes that regulate cell cycle progression and proliferation.



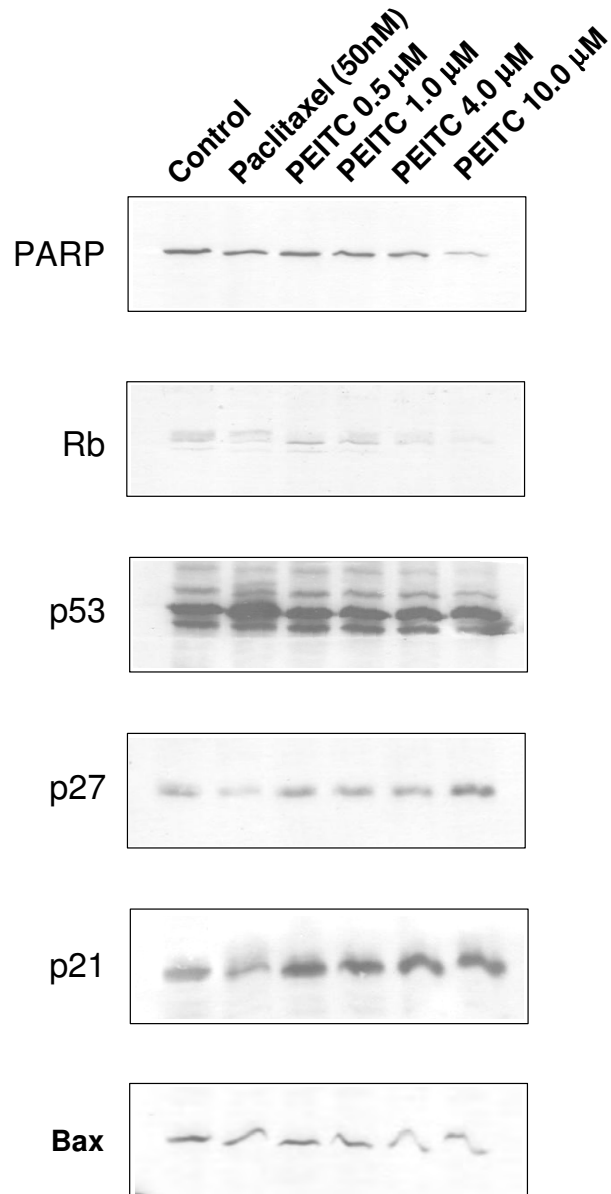


Figure 1. The effects of PEITC on the endogenous levels of indicated proteins in LNCaP cells. LNCaP cells at exponential growth phase were exposed to the indicated concentrations of PEITC for 24 hrs. Paclitaxel was used as a control. The cells were harvested, washed, total proteins extracted and assayed for the protein levels by Western blotting.

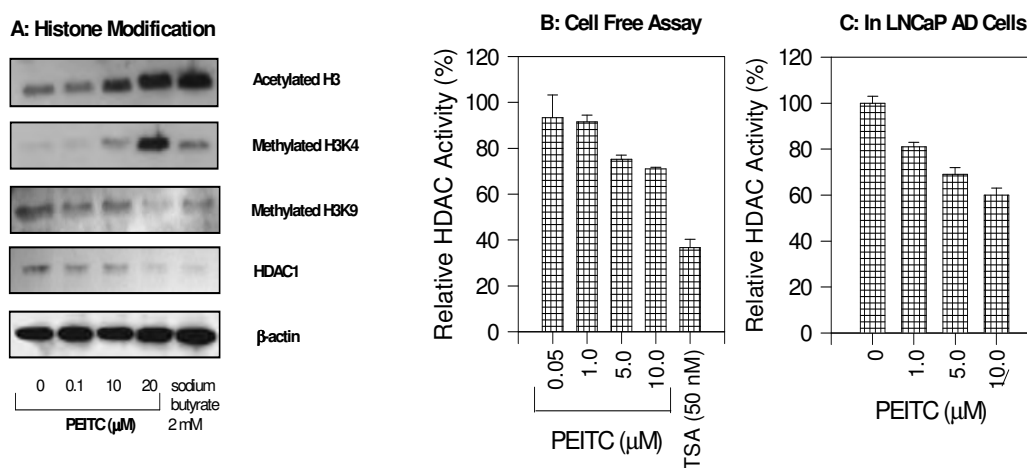


Figure 2. Panel A: Exponentially grown LNCaP cells were exposed for 36 h to various concentrations of PEITC, or to 2 mM of sodium butyrate. The histones were isolated and Western blotting performed using site specific antibodies against histone or  $\beta$ -actin for loading control. The PEITC decreases the expression of HDAC1, increases global histone acetylation, and modifies selective histone methylation that are consistent with the marks of chromatin unfolding and transcription. Panel B: HDAC from HeLa cells were incubated with PEITC at various concentrations and the HDAC activity determined. A known inhibitor of HDAC, trichostatin A (TSA), was used at a concentration pre-determined to achieve about 50% inhibition of the enzyme activity. Results were means + SD of two experiments. Panel C: LNCaP cells after exposure to PEITC for 36 h were examined for HDAC activity using a fluorescent HDAC activity assay (Biomol). Lysates from cells treated with or without PEITC were incubated with HDAC substrate, and the activity compared to a standard curve. The activity was normalized with the control cells without PEITC as 1.0. The results were mean  $\pm$  SD, n=3.

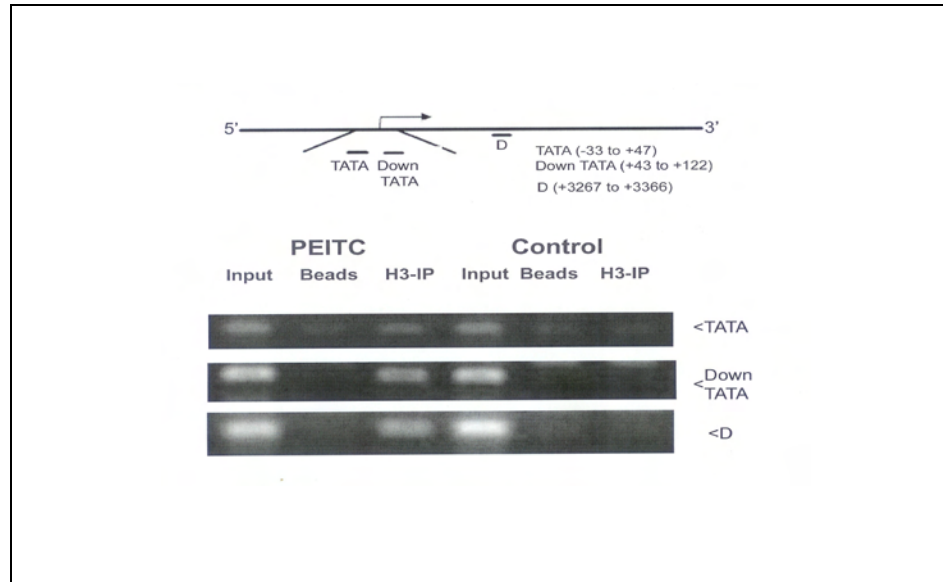


Figure 3. ChIP assay shows association of p21 gene with hyperacetylated histones accumulated by PEITC induction. Schematic representation of PCR primer sets in the upper graph. Lower figure shows chromatin fragments from LNCaP cells after exposure to 10  $\mu$ M PEITC, or to medium without PEITC (Control), were precipitated with an anti-acetylated histone H3 antibody (H3-IP), or with control IG (Beads). Indicated primers for areas of the p21 gene were used for PCR amplification of the DNA isolated from the immunoprecipitated chromatin.

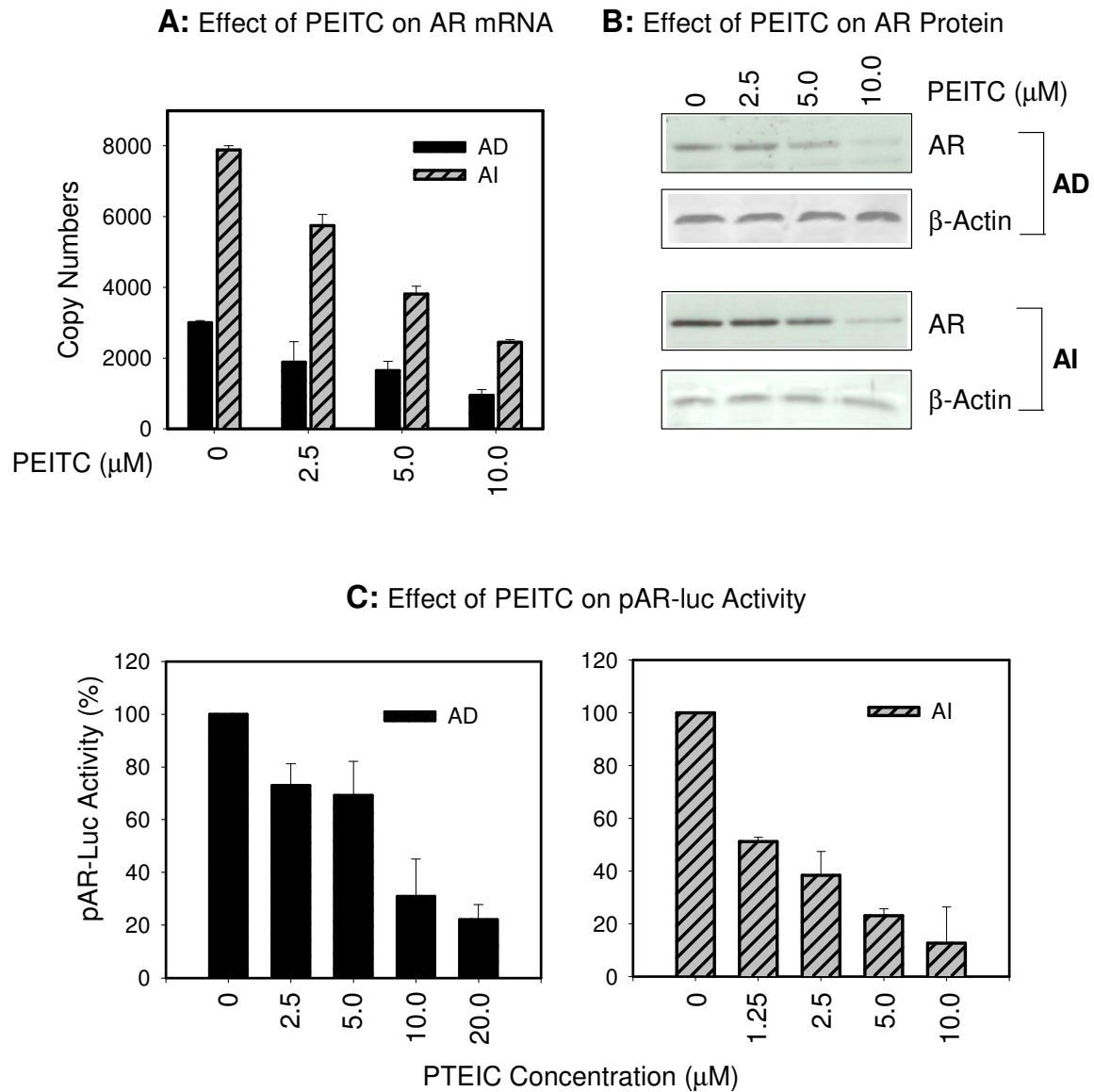


Figure 4. PEITC represses transcription and expression of the androgen receptor (AR). Exposure of androgen-dependent (AD) and a sub-line androgen-independent (AI) LNCaP cells to PEITC resulted in growth inhibition. (A): Repression of AR transcription by PEITC as determined by real-time PCR. (B): Reduction of AR protein expression as determined by Western blotting. (C): Cells transfected with pAR-luc that covers the AR promoter, then exposed to PEITC resulted in decrease of reporter activities. AR repression may be the result of inhibiting the AR promoter.

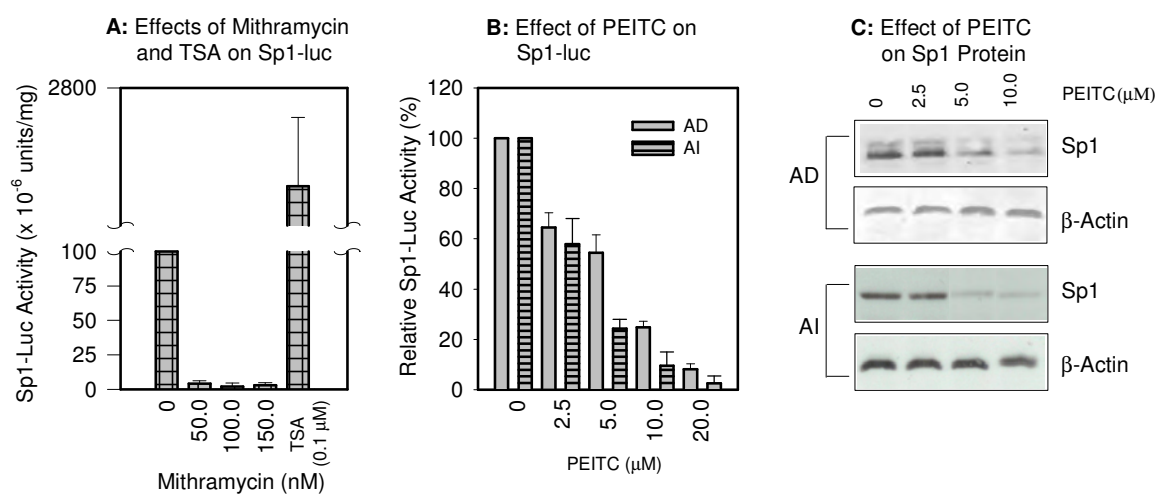
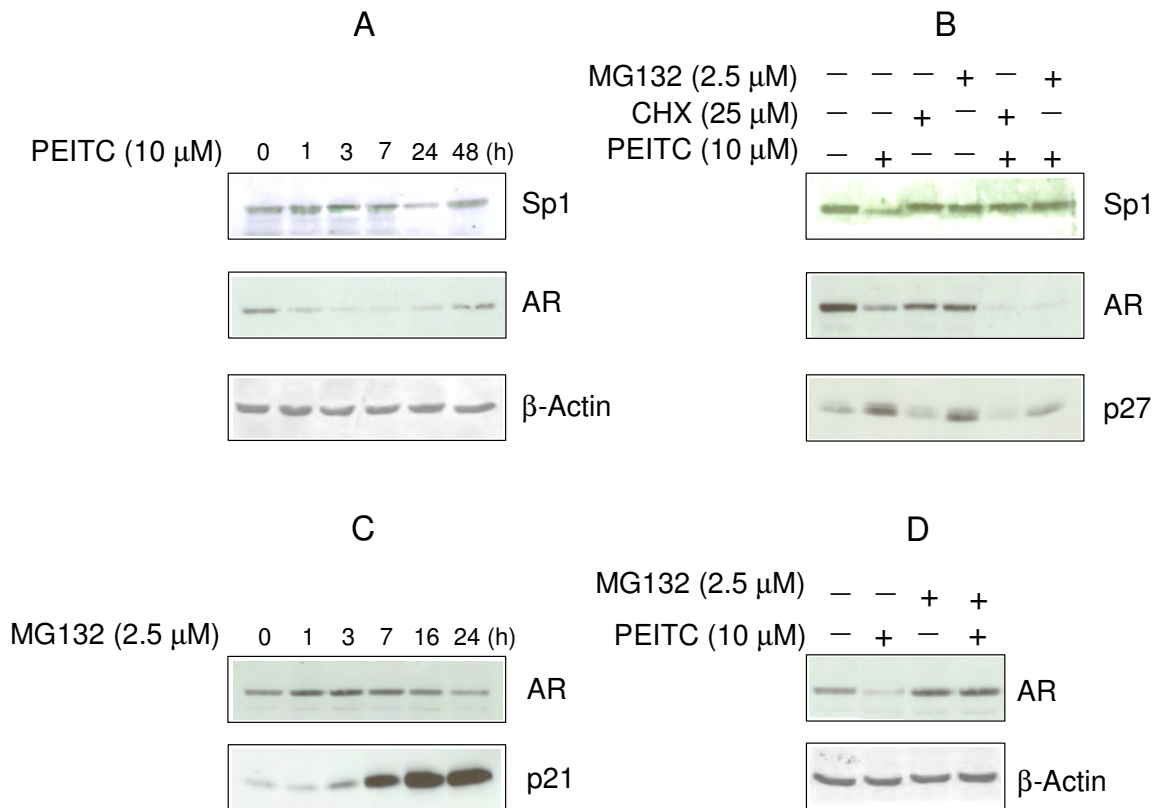


Figure 5. The PEITC effect reducing the activity of Sp1. (A and B): LNCaP AD and AI cells were transfected with pSp1-luc plasmid DNA in a serum-free medium for 24 h. (9). The cells were treated with methramycin or TSA (A), or PEITC (B) for additional 24 h. and the luciferase activity assayed (unit per mg of the proteins). Data represent mean  $\pm$  SD from three independent experiments. (C): AD and AI cells were exposed to PEITC for 24 h and Sp1 level measure by Western blotting.



Figuer 6. Degradation of Sp1 and AR proteins by PEITC. (A) Time course effect of PEITC on Sp1 and AR proteins: The levels of Sp1 and AR of LNCaP AD cells after exposure to 10  $\mu$ M PEITC. (B) Effect of inhibitors of protein synthesis or proteasome on Sp1 and AR degradation by PEITC: AD cells were exposed to PEITC (10  $\mu$ M), CHX( 25  $\mu$ M) or MG-132 (2.5  $\mu$ M) alone, or to PEITC plus CHS or MG-132 for 24 h, and the protein levels of Sp1 and AR determined by Western blotting. (C) Time course effects of proteasome inhibitor on AR degradation; AD cells were exposed to MG-132 (2.5  $\mu$ M) for indicated time period, and the protein levels of AR and p21 measured by Western blotting. (D) AD cells were exposed to PEITC (10  $\mu$ M) for 3 h in the presence or absence of MG-132, and the level of AR protein determined.

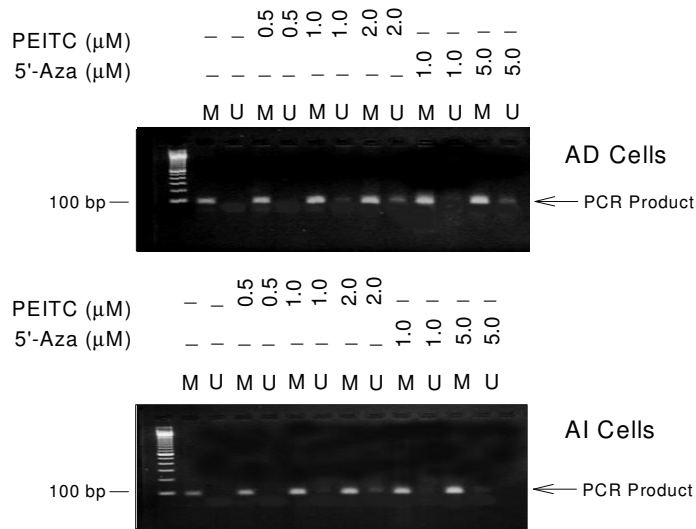


Figure 7. Inhibition of CpG island methylation of GSTP1 by PEITC. LNCaP AD (androgen dependent) and AI (androgen independent) cells grown exponentially were exposed to PEITC for 5 days. The DNA was isolated and the status of methylation measured by MSP (methylation specific PCR) with primers for methylated form (M), or unmethylated form (U). Arrow indicates the PCR product (methylated form: 92 bp, and unmethylated form: 99bp). The unmethylated form of the CpG island became visible in both AD and AI cells after PEITC exposure. This methylation-inhibitory effect was equivalent or stronger than that of cells exposed to 5 μM of 5'-Aza.

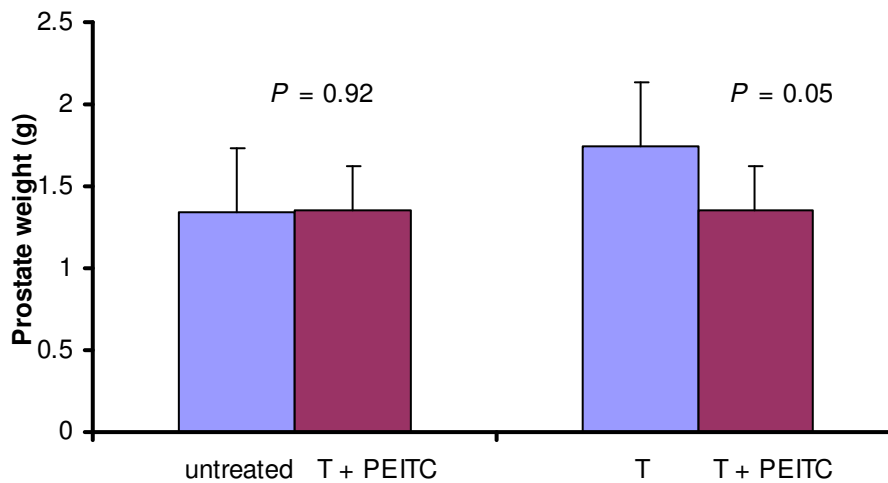


Figure 8. Effects of PEITC on prostate mass in normal rats: administration of PEITC starting at the same time the rats received cyproterone acetate and testosterone and continued until 2 weeks after testosterone. 'Untreated' indicates rats (n=5) received vehicle control of PEITC without cyproterone acetate and testosterone. 'T' indicates rats (n=5) received cyproterone acetate, testosterone and the vehicle control of PEITC. 'T + PEITC' indicates rats (n=7) fed with PEITC, cyproterone acetate and testosterone. Means  $\pm$  SD are depicted.

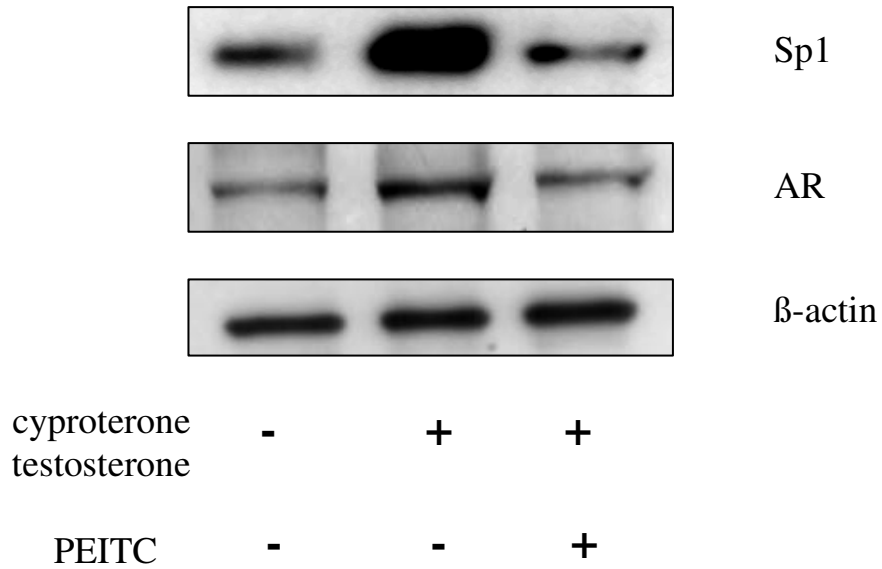


Figure 9. The effects of PEITC on AR and Sp1 expression in the rat prostates. Western blotting of pooled prostates from untreated rats (n=5), rats dosed with cyproterone acetate and testosterone (n=5), and rats fed with PEITC at the same time of cyproterone and testosterone (n=7). The figure shows that AR and Sp1 expression increased after testosterone stimulation, which was negated by PEITC treatment.



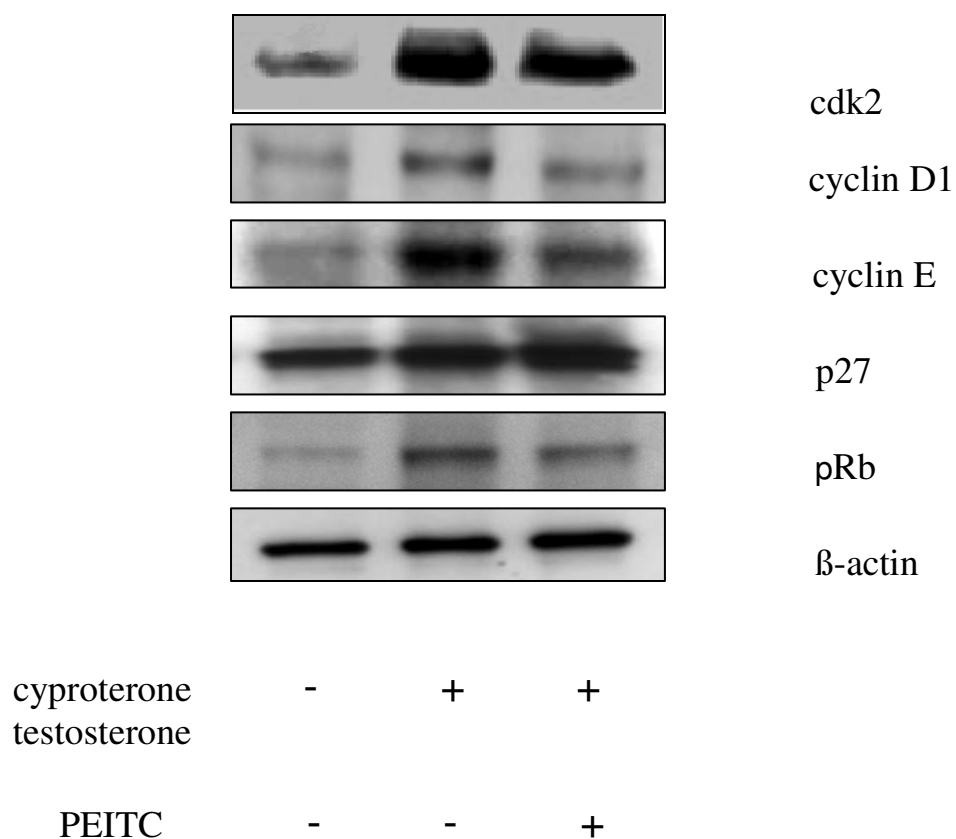


Figure 10. The effects of PEITC on the testosterone-mediated proliferation of the normal prostatic cells in the rats. Western blotting of pooled prostates from untreated control, testosterone-dosed, and PEITC-treated groups show that the testosterone-stimulated cell cycle progression was negated by PEITC, revealed with decrease of cyclins D1 and E, cdk2, and phosphorylated Rb, along with increase of cdk inhibitor p27.

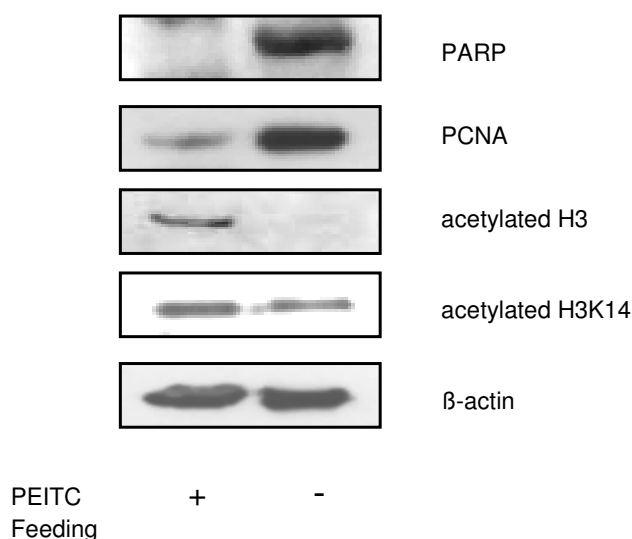


Figure 11. PEITC induced growth arrest and apoptosis in the MNU-induced rat prostate tumors. Western blots were performed with pooled total proteins from the rat prostate tissues with MNU-induced prostate tumors, and the experimental group of rats with induced tumors and treated with PEITC in oral feeding.  $\beta$ -actin was used as a loading control.

## Key research accomplishments

*The presence of epigenetic defects associated with prostate cancer and therapy:* With the investigation of the isothiocyanates and cruciferous vegetables for prostate cancer prevention and therapy, we have demonstrated that the growth regulation of prostate cancer cells by PEITC involves epigenetic regulations. We also showed that genes critically silenced during carcinogenesis could be reversed at the epigenetic level. These findings have in turn demonstrated the presence of the aberrant epigenetic mechanisms that underlining the initiation and progression of prostate cancer. They represent abnormalities at the epigenome that have not been fully described in prostate cancer. For example, there are the abnormalities of local chromatin architecture and histone modifications, and the CpG island methylation of genes. The experimental conclusions may help formulating new diagnostic methods for detecting the aberrant epigenetic defects, and also initiating epigenetic therapy as a targeted approach to correct the abnormalities. The epigenetic therapy could bring the genes back to life, and as a result the cells could be recovered with the full compartment of functions, capable of inhibiting further tumorigenesis. The tumor cells could be reverted to a more normal state, and the side effects of killing normal cells by the conventional chemotherapy could be avoided. In this regard, cruciferous vegetables and isothiocyanates could have an immediate role in combating prostate cancer.

We have demonstrated that isothiocyanates, such as phenethyl isothiocyanate (PEITC), inhibits the growth of prostate cancer cells by targeting cell cycle regulators, including up-regulating p21. PEITC

was found to be a dual inhibitor of histone deacetylases (HDACs) and aberrant CpG island methylation. As a HDAC inhibitor, PEITC modifies histones for transcription competent chromatin to activate p21. We demonstrated, for the first time, that PEITC reverses hypermethylation and reactivates GSTP1 that is silenced in prostate tumors. The silencing of GSTP1 occurs in the vast majority of clinical tumors and is a risk factor. Silencing of GSTP1 and other genes in cell growth and other pathways could similarly be reactivated, thus reversing the cancerous properties of the tumor cells.

We also determined that PEITC represses androgen receptor (AR) via down-regulation of Sp1 and increase protein degradation. The rat bioassay showed that PEITC regulates the activity of testosterone, via down-regulation of AR to modulate the growth of the prostate and prostate tumors. Thus we have demonstrated several molecular targets of PEITC at the epigenetic level, relevant for inhibiting prostate carcinogenesis. They include repressing AR, inhibiting HDACs, inhibiting aberrant CpG island methylation, and activating cell cycle regulators. We have further demonstrated that isothiocyanates, as a class of chemical may have the same activities for preventing and inhibiting other types of cancer. They may be effective at the level of the stem cells thus inhibiting the hormone refractory prostate cancer, which should be further evaluated.

### **Reportable outcomes:**

1. Chiao, J. W., Beklemisheva, A, Yang, J, Feng, Y, Dai, W, Liu, D., Wang, LG, Transcriptional activation of p21 by phenethyl isothiocyanate via chromatin remodeling and c-myc de-repression in prostate cancer cells. *Am Assoc Canc Res.*47, 1314, 2006.
2. Beklemisheva, A, Fang, Y, Feng, J, Ma, X, and Chiao, JW, Epigenetic mechanism of growth inhibition induced by phenylhexyl isothiocyanate in prostate cancer cells. *Anticancer Research* 26, 1225, 2006.
3. Beklemisheva, A, Feng, J, Yeh, YA, Wang, LG and Chiao, JW, Modulating testosterone stimulated prostate growth by phenethyl isothiocyanate via Sp1 and androgen receptor down-regulation. *The Prostate*, 67, 863, 2007.
4. Wang, LG, Beklemisheva, A, Liu, XM, Ferrari, AC, Feng, J, and Chiao, JW. Dual action on promoter demethylation and chromatin by an isothiocyanate restored GSTP1 silenced in prostate cancer. *Mol. Carcinogenesis*. 46, 24, 2007.
5. Wang, LG, Liu, XM. And Chiao, JW., Repression of androgen receptor cancer cells by phenethyl isothiocyanate. *Carcinogenesis*. 27. 2124. 2006.
6. Ma, X, Fang Y, Beklemisheva A, Dai W, Feng J, Ahmed T, Liu D and Chiao JW. Phenylhexyl isothiocyanate inhibits histone deacetylases and remodels chromatin to induce growth arrest in human leukemia cells. *Int J Oncology* 28, 1287, 2006.
7. Lu L, Liu D, Ma X, Beklemisheva, Seiter K, Ahmed T and Chiao JW. The phenylhexyl isothiocyanate induces apoptosis and inhibits leukemia cell growth in vivo. *Oncology Reports* 16, 1363, 2006.

8. Chiao JW, Wu H, Ramaswamy G, Conaway CC, Chung FL Wang L, and Liu D. Ingestion of an isothiocyanate metabolite from cruciferous vegetables inhibits growth of human prostate cancer cell xenografts by apoptosis and cell cycle arrest. *Carcinogenesis* 25, 1403, 2004.
9. Wang, LG, Feng, J, Liu, XM, Beklemisheva, A, and Chiao, JW, Phenethyl isothiocyanate-mediated cross talk between chromatin remodeling and DNA demethylation- restoring GSTP1 expression in prostate cancer. *Am Assoc Canc Res.* 46, 1226, 2005.

## Conclusion

The major implication of our findings is that PEITC mediates regulations at the epigenetic level for prostate cancer chemoprevention and chemotherapy. Evidence is in turn obtained that aberrant epigenetic regulation exists as a critical pathogenic mechanism of prostate cancer.

We have obtained experimental proofs supporting our hypothesis that phenethyl isothiocyanate (PEITC), present naturally in cruciferous vegetables, inhibits the growth of prostate cancer cells by targeting cell cycle regulators, such as p21. The up-regulating p21 involves an epigenetic mechanism. PEITC was discovered as a HDAC inhibitor, enhancing acetylation of histones, and selectively modifying histone methylation, thus remodeling chromatin for transcriptional activation of p21.

We also discovered that PEITC represses the androgen receptor (AR), and inhibits the growth of both androgen-dependent and –independent human prostate cancer cells. At transcriptional level the AR was reduced via inhibition of the transcription factor Sp1, and at post-translational level by accelerating protein degradation. The regulation of cell cycle and the AR attenuate the uncontrolled growth of prostate cancer cells. The epigenetic regulation represents the primary mechanism of PEITC for the growth attenuation.

We further discovered that PEITC is an inhibitor of aberrant CpG island methylation. As a dual inhibitor of aberrant CpG island methylation and also HDACs, we demonstrated that PEITC reverses hypermethylation and reactivates GSTP1, which is a detoxifying enzyme, critically inactivated in the vast majority of prostate tumors. Since inactivation of GSTP1 represents a critical event in prostate carcinogenesis, reactivation of GSTP1 by PEITC means the correction of a key cancerous property and that is highly significant, revealing the basis for both prevention and treatment of prostate cancer by PEITC. The epigenetic approach with the reactivation of silenced genes allows the recovery of key gene functions in the cells, which could inhibit further tumorigenesis.

Our investigation has revealed several relevant molecular targets of PEITC for prostate cancer prevention and therapy. They include repressing AR, inhibiting HDACs, inhibiting aberrant CpG island methylation, and activating cell cycle regulators. As demonstrated by the bioassay, PEITC regulates the activity of testosterone on AR for the growth of the prostates. Our findings have revealed the basis of PEITC as a practical chemopreventive and therapeutic agent for prostate cancer. Isothiocyanates as a class of chemicals may possess the same activities as PEITC, which may include an inhibitory effect on prostate stem cells that needs to be further investigated.

## References

1. Chiao, J. W., Beklemisheva, A, Yang, J, Feng, Y, Dai, W, Liu, D., Wang, LG, Transcriptional activation of p21 by phenethyl isothiocyanate via chromatin remodeling and c-myc de-repression in prostate cancer cells. *Am Assoc Canc Res.*47, 1314, 2006.
2. Beklemisheva, A, Fang, Y, Feng, J, Ma, X, and Chiao, JW, Epigenetic mechanism of growth inhibition induced by phenylhexyl isothiocyanate in prostate cancer cells. *Anticancer Research* 26, 1225, 2006.
3. Beklemisheva, A, Feng, J, Yeh, YA, Wang, LG and Chiao, JW, Modulating testosterone stimulated prostate growth by phenethyl isothiocyanate via Sp1 and androgen receptor down-regulation. *The Prostate*, 67,863, 2007.
4. Wang, LG, Beklemisheva, A, Liu, XM, Ferrari, AC, Feng, J, and Chiao, JW. Dual action on promoter demethylation and chromatin by an isothiocyanate restored GSTP1 silenced in prostate cancer. *Mol. Carcinogenesis*. 46, 24, 2007.
5. Wang, LG, Liu, D, Ahmed, T, Chung, FL, Conaway, c, and Chiao, JW. Targeting cell cycle machinery as a molecular mechanism of isothiocyanate in prostate cancer prevention. *Int. J. Oncology*, 24, 187, 2004.
6. Chiao, JW, Wu, H., Ramaswamy, H, Conaway, C, Chung, FL, Wang, LG and Liu, D. Ingestion of an isothiocyanate metabolite from cruciferous vegetables inhibits growth of human prostate cancer cell xenografts by apoptosis and cell cycle arrest. *Carcinogenesis* 25, 1403, 2004.
7. Wang, LG, Feng, J, Liu, XM, Beklemisheva, A, and Chiao, JW, Phenethyl isothiocyanate-mediated cross talk between chromatin remodeling and DNA demethylation- restoring GSTP1 expression in prostate cancer. *Am Assoc Canc Res*. 46, 1226, 2005.
8. Chiao, JW., Chung, FL., Krzeminski, J., Amin, S., Arshad, R., Ahmed, T. and Conaway,CC. Modulation of growth of human prostate cancer cells by the N-acetylcysteine conjugate of phenethyl isothiocyanate. *Int J. Oncology* 16, 1215, 2000.
9. Wang, LG., Liu, XM and Chiao, JW, Repression of androgen receptor in prostate cancer cells by phenethyl isothiocyanate. *Carcinogenesis*. 27, 2124, 2006.
10. Ma, X, Fang Y, Beklemisheva A, Dai W, Feng J, Ahmed T, Liu D and Chiao JW. Phenylhexyl isothiocyanate inhibits histone deacetylases and remodels chromatin to induce growth arrest in human leukemia cells. *Int J Oncology* 28, 1287, 2006.
11. Lu L, Liu D, Ma X, Beklemisheva A, Seiter K, Ahmed, T and Chiao J.W., Phenylhexyl isothiocyanate induces apoptosis and inhibits leukemia cell growth in vivo. *Onocology Reports*. 16, 1363, 2006.

## **Appendices**

This is a revised report and hard copies of seven manuscripts were sent with the previous report. Two missed AACR abstracts of 2005 and 2006 are attached herein.

**#5591** Transcriptional activation of p21 by phenethyl isothiocyanate via chromatin remodeling and c-myc de-repression in prostate cancer cells. Jen Wei Chiao, Anastasia Bekdemisheva, J. Yang, Y. Feng, Wei Dai, D. Liu, L. G. Wang, X. M. Liu. *New York Medical College, Valhalla, NY and New York University School of Medicine, New York, NY.*

The isothiocyanates, present naturally in cruciferous vegetables are potent chemopreventive agents for carcinogen-induced cancers in rodents. The major action is thought to be cytoprotection by inhibiting the metabolism of procarcinogens and inducing phase 2 enzymes to block the initiation of carcinogenesis. We have demonstrated that the isothiocyanates are also growth regulators, inhibiting the cell cycle cdk activity and/or up-regulating the inhibitor p21<sup>WAF1</sup> (p21) in cancer cells. The up-stream mechanism to modulate cell cycle progression remained to be elucidated. Here we demonstrate that exposure of human prostate cancer cells LNCaP to phenethyl isothiocyanate (PEITC) resulted in G1 arrest and p21 induction. The hypothesis that PEITC inhibits cell growth via chromatin remodeling was investigated. We demonstrated with a cell-free assay, that PEITC inhibited the activity of histone deacetylases (HDAC), the enzyme acetylates the histones. Additionally the level of HDAC was reduced. The PEITC-exposed LNCaP cells were shown to have reduced HDAC activity. Analyses of the status of histone acetylation revealed a significant enhancement of global acetylation, including the acetylation of histones H3 and H4, and selective acetylation of H3K14. These are marks consistent to transcription competent chromatin. To further examine the relationship between acetylated histones and p21 activation, ChIP assay was performed. Using an anti-acetylated histone H3 antibody, we demonstrated for the first time that the chromatin from cells exposed to PEITC contained more p21 DNA in the precipitates of hyperacetylated histones, as compared with cells without PEITC. This indicates the possible increases of accessibility of transcriptional machinery to the p21 promoter after chromatin unfolding. The investigation further revealed that the c-myc expression was significantly down-regulated by PEITC that paralleled the inhibition of HDAC. The decreased expression of c-myc may further release p21 transcription otherwise repressed by the c-myc oncogene. The experimental results indicate that although PEITC affects multiple molecular pathways, regulation of the epigenetic targets may be important mechanisms leading to the up-regulation of p21, thereby mediating cell cycle arrest in prostate cancer cells. Thus, our observations have provided a critical aspect for further investigating PEITC and other isothiocyanates in chemoprevention and inhibiting the growth of malignant cells.



#5193 **Phenethyl isothiocyanate-mediated cross talk between chromatin remodeling and DNA demethylation-restoring GSTP1 expression in prostate cancer.** Longgui Wang, J. Feng, X. M. Liu, A. Beklemisheva, J. W. Chiao. *New York University School of Medicine, New York, NY and New York Medical College, Valhalla, NY.*

The basis of phenethyl isothiocyanate (PEITC), a natural component of cruciferous vegetables, for restoring silenced p1-class glutathione S-transferase (GSTP1) expression in prostate cancer via cross talk between histone modification and DNA demethylation has been investigated. Isothiocyanates occur naturally as thioglucoside conjugates, i.e., glucosinolates, in a wide variety of cruciferous vegetables. Hydrolysis of the glucosinolate gluconasturtin yields PEITC. Isothiocyanates have been shown to have potent cancer chemopreventive activity in experimental cancer models in rodents. The mode of action has been associated with cytoprotection, i.e. blocking the metabolism of procarcinogens with phase 1 enzymes and inducing phase 2 enzymes such as glutathione S-transferase, etc. to facilitate carcinogen excretion. Our research has demonstrated that isothiocyanates such as PEITC and their metabolites also induce growth arrest and apoptosis in human prostate cancer cells. Their effects to inhibit post-initiation progression of carcinogenesis revealed. To investigate the molecular mechanism of blocking prostate carcinogenesis, the effects of PEITC on histone remodeling and DNA methylation were studied with prostate cancer LNCaP cells. The exposure to PEITC decreased the activity of histone deacetylases, the enzymes that remove the acetyl groups. As a result, the global levels of acetylation of histone H3 were increased significantly in a concentration-related manner. Compared to untreated control cells, exposure to PEITC also increased methylation of histone H3 lysine 4, and decreased methylation of histone H3 lysine 9. This selective acetylation/methylation of histones is consistent to the marks of activated chromatin. The status of methylation of CpG island at the promoter of GSTP1 was analyzed with methylation specific PCR. Hypermethylation of the CpG island at the promoter of GSTP1 is commonly present in prostate tumors from majority of patients. It is a genomic abnormality and silencing of the associated genes by gene promoter hypermethylation has been considered as a pathogenic cause of the disease. In the untreated control cells there was no basal level of the un-methylated form of CpG island of GSTP1. After exposure to PEITC the un-methylated form of GSTP1 was increased significantly in a concentration-dependent manner. The effects of demethylation by PEITC were similar to that of 5'-Aza-2'-deoxycytidine, a demethylation agent that was used as a positive control. The findings are significant since they suggested that the aberrant DNA hypermethylation of cancer cells can be corrected. The restoration of the silenced gene expression by PEITC-mediated cross talk between histone modifications and DNA demethylation represent an epigenetic regulation that mediates the inhibition of initiation and progression of prostate cancer.